Case Report

Paradoxical Adipose Hyperplasia and Cellular Effects After Cryolipolysis: A Case Report

Scott A. Seaman, BS; Shruti C. Tannan, MD; Yiqi Cao, BS; Shayn M. Peirce, PhD; and Thomas J. Gampper, MD

Abstract

Cryolipolysis is a noninvasive technique for the reduction of subcutaneous adipose tissue by controlled, localized cooling, causing adipocyte apoptosis, reportedly without affecting surrounding tissue. Although cryolipolysis has a low incidence of adverse side effects, 33 cases of paradoxical adipose hyperplasia (PAH) have been reported and the precise pathogenesis of PAH is poorly understood. This present case study of PAH aims to characterize the pathological changes in the adipose tissue of PAH on a cellular level by using multiple different assays (hematoxylin and eosin staining, LIVE/DEAD staining, BODIPY® 558/568 C12 (4,4-Difluoro-5-(2-Thienyl)-4-Bora-3a,4a-Diaza-s-Indacene-3-dodecanoic acid) staining) to identify the underlying mechanism of PAH and reduce the prevalence of PAH in the future. Tissue with PAH had fewer viable cells, significantly decreased quantities of interstitial cells (p = 0.04), and fewer vessels per adipose tissue area when compared to the control tissue. Adipocytes from the PAH tissue were on average slightly smaller than the control adipocytes. Adipocytes of PAH tissue had irregularly contoured edges when compared to the smooth, round edges of the control tissue. These findings from a neutral third party are contrary to prior reports from the inventors of this technique regarding effects of cryolipolysis on both the microvasculature and interstitial cells in adipose tissue. Our use of different assays to compare cryolipolysis-treated PAH tissue with untreated adipose tissue in the same patient showed adipose tissue that developed PAH was hypocellular and hypovascular. Contrary to prior reports from the inventors, cryolipolysis may cause vessel loss, which could lead to ischemia and/or hypoxia that further contributes to adipocyte death.

Level of Evidence: 5

Accepted for publication May 7, 2015; online publish-ahead-of-print November 20, 2015.

Cryolipolysis is a noninvasive technique for the reduction of subcutaneous adipose tissue by controlled, localized cooling.1,2 During this procedure, a fold of the adipose tissue is drawn between two cooling plates, and the temperature is lowered to 0°C. Cold exposures are well tolerated and result in fat loss without causing significant skin injury.3,4 This procedure reportedly triggers adipocyte apoptosis without affecting surrounding tissue.1,2 Histologic studies of porcine preclinical models show that cryolipolysis induces an inflammatory response in which adipocytes become surrounded by neutrophils, macrophages, and other phagocytic cells.3 The procedure results in decreased size and disaggregation of adipocytes, which leads to a reduction in the thickness of the subcutaneous fat tissue.3 Jalian et al6 report that as of 2014, there have been 650,000 cryolipolysis treatments worldwide since the introduction of the procedure.

A blinded comparison of pre-treatment photos and post-cryolipolysis treatment photos of 50 subjects showed that physician reviewers were able to differentiate the two sets of photos.
photographs 92% of the time, demonstrating the efficacy of cryolipolysis. In addition, a pilot study in which six patient abdominal subcutaneous fat deposits were treated with cryolipolysis revealed that after 6 months the clinical treatment group showed an average of 19.6% fat reduction by ultrasound imaging. The American Society for Aesthetic Plastic Surgery ranks nonsurgical fat reduction as the tenth most frequent nonsurgical cosmetic procedure and demonstrates its growing popularity by citing a 42.7% increase in nonsurgical fat reduction procedures, from 94,922 in 2013 to 135,448 in 2014.

Cryolipolysis is generally considered a safe procedure, with side effects such as edema, bruising, erythema, and transient neuralgia, and these side effects are typically self-resolving within 2 weeks posttreatment. Stevens et al examined medical records for over 500 patients treated with cryolipolysis and found that only three patients reported mild to moderate pain or neuralgia, and no serious adverse events were reported. Although cryolipolysis has a low incidence of serious adverse side effects, 33 cases of paradoxical adipose hyperplasia (PAH) (out of roughly 650,000 cryolipolysis treatments) have been reported to device manufacturers. Derrick et al mined the literature for articles published on cryolipolysis and found clinical reports for 1445 patients, with only two of these patients (0.14%) reporting PAH. In these rare cases of PAH, the adipose tissue at the treatment site is reported to increase in mass to a degree that is clearly visible at the macroscopic scale. The precise pathogenesis of PAH is not well understood, with only few studies examining this phenomenon. Jalian et al reported a case study of enlarged tissue at the treatment site and examined this hyperplastic tissue at the microscopic and macroscopic level. They used two modalities—magnetic resonance imaging (MRI) and hematoxylin and eosin (H&E) staining—to study macroscopic and microscopic differences in PAH tissue as compared to untreated tissue. MRI data revealed growth of the cryolipolysis-treated tissue, while H&E staining revealed decreased adipocyte organization, septal thickening around fat globules, and an increase in vascularity in the cryolipolysis-treated tissue.

This present case study aims to characterize the pathological changes in the adipose tissue of PAH on a cellular level by using multiple assays (H&E staining, LIVE/DEAD staining, BODIPY staining), an important step towards identifying the underlying mechanism of PAH and reducing the prevalence of PAH in the future.

**CASE REPORT**

The patient is a 48-year-old female who underwent two total cryolipolysis treatments with the CoolSculpting device (Zeltiq, Pleasanton, CA) in back-to-back months. Both treatments were performed to her abdomen, posterior trunk, and bilateral flanks. She had developed palpable firmness and a visible increase in the adipose tissue of her abdomen and posterior trunk 3 months after the second cryolipolysis treatment (Figure 1). These clinical findings remained unchanged 6 months after the second cryolipolysis treatment and were consistent with PAH. Six months after the second cryolipolysis treatment, the patient presented to our office in November of 2013 and subsequently elected to undergo suction lipectomy of these hyperplastic areas. Suction lipectomy was performed 7 months after the second cryolipolysis treatment (1 month after presenting to our office). At the time of the procedure, adipose tissue from both her previously-treated anatomic areas with PAH and from untreated deposits (control tissue) were sampled by direct open excision. Analyses of the tissue samples were performed by researchers blinded to the tissue source (cryolipolysis-treated tissue with subsequent PAH versus untreated, control tissue). Tissue was acquired according to an approved protocol by the Institutional Review Board at The University of Virginia.

Small (~5 mm), excised tissue samples from control and cryolipolysis-treated areas with PAH were stained with a LIVE/DEAD kit (Life Technologies L-7013, Grand Island, NY) to assess interstitial cell viability and total number of interstitial cells (Figure 2A and B). Briefly, Components A and B of the LIVE/DEAD kit were diluted 1:250 in phosphate-buffered saline (PBS) and 100 μL of staining solution was added to each sample tube and incubated at room temperature for 30 minutes, protected from light. Samples were washed three times with PBS and were mounted on gelatin-coated slides for imaging. All samples were imaged using a Nikon (Melville, NY) TE 2000-E2 microscope equipped with a confocal attachment. Viable cells stained green and dead, nonviable cells stained red, allowing for quantification of percent of viable cells and total number of cells. Representative images (200× magnification, 40 μm Z-stacks) and quantification of three unique fields of view (FOV) per sample reveal a lower percentage of viable cells in the tissue with PAH when compared to the control tissue (Figure 2C). In addition, the total number of interstitial cells (which include vascular cells, immune cells, and perivascular cells) in the tissue with PAH is significantly decreased (p = 0.04) when compared to the control tissue (Figure 2D).

Separate, small (~5 mm), excised tissue samples from control and cryolipolysis-treated areas with PAH were fixed with 4% (volume/volume) paraformaldehyde and incubated overnight at 4°C. The number of intact adipocytes for each tissue sample was assessed using a lipid specific stain, BODIPY 558/568 (Life Technologies D-2219, Grand Island, NY), and manual counting of adipocytes that appeared intact (as defined by absence of lipid droplets and a spherical shape consistent with untreated adipose tissue). Briefly, samples were washed three times following paraformaldehyde fixation and were incubated in 100 μL of 10 μg/mL BODIPY/PBS solution for 20 minutes at 37°C, protected from light. Adipose tissue samples were also stained concurrently with isoelectin GS-IB4 AlexaFluor 647 (1:200 dilution, Life Technologies...
I32450, Grand Island, NY), which binds to endothelial cells, to visualize the extent of vascularization in the adipose tissue (Figure 3). The isolectin stain was incubated overnight at 4°C, protected from light. Samples were washed three times with PBS and were mounted on gelatin-coated slides for imaging. Confocal microscopy was used to acquire 200× magnification, 40 μm thick Z-stacks of four unique FOVs per sample. Representative images (Figure 3A and B) and quantification of these images (Figure 3C) via manual counting of intact adipocytes revealed similar numbers of intact adipocytes per FOV in adipose with PAH and untreated adipose. It was qualitatively apparent in these images that the tissue with PAH had fewer blood vessels (less isolectin staining) than the control tissue (Figure 3A and 3B).

Figure 1. Pretreatment photos (A, C, E, G, I, and K) and photos taken 4 months after the patient’s second cryolipolysis treatment (B, D, F, H, J, and L) are shown. Posttreatment growth of adiposity is seen in the posterior brassiere-line and the abdomen when compared to pretreatment photos of the 48-year-old woman. The entire abdomen, posterior trunk brassiere-line, and flanks were treated with cryolipolysis according to manufacturer’s guidelines. Photographs courtesy of B Straka.
Additional control and cryolipolysis-treated samples were processed for H&E staining to visualize excised fat in cross sections in order to quantify vessel density and adipocyte area (Figure 4). Multiple images were acquired at 100× magnification using a Nikon TE 2000-E2 microscope and montaged together to provide a complete image of the tissue slice. The entire area of the montage analyzed for the control tissue was 14.00 mm² and the cryolipolysis-treated tissue was 4.87 mm² (data not shown). Arrows in Figure 4 indicate examples of vessels that were counted. The number of vessels per adipose tissue per montage area was increased in the control tissue (1.143 vessels/mm² adipose tissue) when compared to the tissue with PAH (0.616 vessels/mm² adipose tissue), consistent with observations of the BODIPY- and isolectin-stained tissues (Figure 3). The cross-sectional areas of 20 adipocytes were measured. One 100× image of the montage for control and PAH tissue was selected, in which the entire FOV contained adipocytes. The areas of 20 adipocytes in these images of control and cryolipolysis-treated tissue were quantified, starting in the bottom left corner of the 100 × image and progressing to the top right corner of the image until 20 adipocytes were quantified. The adipocytes from the PAH tissue were, on average, slightly smaller than the control adipocytes (Figure 4E). The average number of adipocytes per area was also quantified, and there was no significant difference between the number of adipocytes per area in control and cryolipolysis-treated tissues (Figure 4F). Interestingly, individual adipocytes in the tissue with PAH appeared to have irregularly contoured edges (Figure 4B and D) when compared to the smooth, round edges of the control tissue (Figure 4A and C). These irregular surfaces (possible plasma membrane blebbing) may

Figure 2. LIVE/DEAD staining reveals a decrease in the percent of viable interstitial cells and total number of interstitial cells in cryolipolysis-treated tissue when compared to control tissue. Tissue samples from (A) control and (B) cryolipolysis-treated tissue were stained with a LIVE/DEAD kit to assess (C) interstitial cell viability and (D) total number of interstitial cells. Representative images and quantification of three fields of view (FOV) reveal a lower percentage of viable cells in the cryolipolysis-treated tissue when compared to the control tissue (C) and a statistically significant decrease in the total number of interstitial cells in the cryolipolysis-treated tissue (D). A two-tailed paired t-test was used to evaluate statistical significance (statistical significance asserted at p-value < 0.05) for both quantification methods. Scale bar = 50 μm.
be the preceding step to necrosis or apoptosis\textsuperscript{10} and would be consistent with the primary mechanism of cryolipolysis treatment: the induction of apoptosis.\textsuperscript{1}

**DISCUSSION**

Cryolipolysis is being increasingly used in both plastic surgery and nonsurgical subspecialties as a noninvasive alternative to surgical treatment of excess adipose tissue.\textsuperscript{2-4,9} The technique was initially approved in 2008 by the United States Food and Drug Administration for treatment of focal adiposity of the flanks, and then expanded to abdominal lipoatrophy in 2011. This innovation represents the marriage of a thoughtful observation—the recognition of fat atrophy from “popsicle panniculitis,” an often-treated entity by dermatologists—with the translation of that clinical observation into a treatment modality.

The published mechanism of cryolipolysis is selective cell death of adipocytes following cold exposure.\textsuperscript{1} The adipocytes crystallize, undergo apoptosis, and are then eliminated by macrophage engulfment after approximately 3 months.\textsuperscript{1} The inflammatory process is thought to peak at 2-4 weeks and be complete by the 3 month mark.\textsuperscript{11} CoolSculpting treatments are reportedly specific to adipocytes, and, per the inventors and device manufacturers, do not affect surrounding cells or tissues. However, mechanisms of cell death and eventual elimination are not well understood.\textsuperscript{7} PAH is a rare adverse event after cryolipolysis, and has not been studied extensively. Jalian et al\textsuperscript{6} report a case of PAH and found decreased adipocyte organization and an increase in vascularity of PAH tissue when compared to control tissue after observation using H&E staining and MRI.

Interestingly, our findings are contrary to prior reports from the inventors of this technique regarding both the vascular changes associated with PAH, as well as the...
deleterious effects of cryolipolysis on interstitial cells in adipose tissue.\textsuperscript{5,6,8,9} Our use of different assays to compare cryolipolysis-treated PAH tissue with untreated adipose tissue in the same patient showed that the adipose tissue that developed PAH was both hypocellular and hypovascular. Specifically, our results of the LIVE/DEAD kit imaging reveal a decrease in the number of viable interstitial cells in tissue with PAH following cryolipolysis treatment. These interstitial cells include macrophages, endothelial cells, adipose-derived stem cells, and blood-derived cells, and constitute a complex niche within the adipose tissue that plays a role in adipose tissue remodeling.\textsuperscript{12} To confirm these findings, an endothelial cell–specific stain and high magnification quantification of H&E stains were performed to quantify vascularity in the different adipose tissue samples and we found that the cryolipolysis-treated tissue with PAH was less vascularized than control tissue. Our studies did not reveal significant changes in adipocyte areas, adipocytes per area, or the number of intact adipocytes.

Although the exact mechanisms for adipocyte death and elimination following cold exposure are not known, previous studies suggest that cold exposure exclusively triggers adipocytes apoptosis.\textsuperscript{7} Our study suggests that in PAH cases, the low temperatures may have deleterious effects on the vascular cells in the adipose tissue, which may cause complications during adipose tissue recovery. Hence, it is possible that, in addition to having direct effects on adipocytes, cryolipolysis may also cause blood vessel loss,

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Hematoxylin and eosin staining reveals no significant difference in average adipocyte area or adipocytes per area. Vessels were counted (arrows) and the number of vessels per adipose tissue area was increased in the (A, C) control tissue when compared to the (B, D) cryolipolysis-treated tissue (data not shown). (B, D) Cryolipolysis-treated tissue adipocytes were on average slightly smaller than the (E) control tissue adipocytes, with similar numbers of adipocytes per area in both control and (F) cryolipolysis-treated tissues. All statistical tests: two-tailed paired t-test, statistical significance asserted at \( p \)-value < 0.05. Scale bar = 50 \( \mu \)m.}
\end{figure}
which could lead to ischemia and/or hypoxia that further contributes to adipocyte death. However, in our study, hypocellular PAH tissue was accompanied by a hypovascular state, as would be expected.

Although PAH is characterized by the macroscopic appearance of adipose tissue enlargement upon clinical presentation, our data suggest that the microstructure of PAH tissue may be hypovascular and hypocellular. It is counterintuitive that a decrease in vascularity and interstitial cells could be commensurate with clinically enlarged adipose deposits in PAH tissue after cryolipolysis. One would expect that an enlarged adipose depot would be accompanied or enabled by increased vessel density because a larger, more hyperplastic adipose tissue depot would require additional delivery of nutrients and oxygen.

Though it is believed that PAH clinically presents itself with an enlarged mass of adipose tissue, it is difficult to measure the actual mass of the cryolipolysis-treated area, both before and after the procedure. In our study, it may be possible that there was a decrease in adipose tissue volume peripheral to where the cryolipolysis applicator was applied. This may cause a perceived increase in volume in the region of adipose that was in contact with the center of the cryolipolysis applicator, which would be consistent with the prior observations of PAH. MRI images helped visualize the PAH region in the study by Jalian et al., but neither our study nor theirs have not been able to provide an explanation for this perceived increase in mass. A case report of PAH presented by Stefani postulates that the negative suction process of the cryolipolysis instrument may stimulate adipocyte proliferation [similar to the effect of the BRAVA external breast tissue expander (Brava, LLC; Miami, FL) system on the breast], which leads to a paradoxical increase in adipose tissue. In our study, the patient presented with palpable stiffening of the affected adipose tissue, in conjunction with a perceived increase in adipose mass. Although the cause of this stiffening is unclear, we surmise that it may be due to fibrosis from the less vascularized, more hypoxic affected adipose tissue. In response to hypoxia/ischemia, resident fibroblasts rearrange the extracellular matrix and produce collagen, two hallmarks of fibrosis, in adipose tissue, renal tissue, and cardiac tissue. It is possible that this potential fibrosis caused the adipocytes within the affected PAH area to take on irregular formation (membrane blebbing, abnormal edges) in response to the changing extracellular matrix. It is also possible that the reported increased mass of PAH may be due to this fibrosis of the adipose tissue, in which the extracellular matrix is expanding. The notion that fibrosis is the cause of observed PAH is speculative at this point, and further studies are needed to confirm whether PAH may be attributed to fibrosis of the adipose tissue.

Our studies analyzed the PAH tissue at a later timepoint than the Jalian et al. report (7 months vs. 3 months posttreatment), and it is possible that these two studies observed different stages on the spectrum of PAH. Since adipose tissue is a dynamic microenvironment with the capability to expand and decrease in size, this process is best understood as a continuum of changing cellular events. Jalian et al. may have reported the early stages of PAH (aggressive adipose tissue hyperplasia, increased angiogenesis/vessel ingrowth) and our studies may have captured the later stages (adipocyte apoptosis or homeostasis, reduction of vessel ingrowth and angiogenesis, ceasing of hyperplastic process). This differing time of examination may explain the discrepancy in vascularity between the two studies. The effects of cryolipolysis and its induction of PAH may not be fully captured in the assays performed in our study or the Jalian et al. study. Although our studies have focused on vascularity and adipocyte changes, there may be other cells (such as macrophages, tissue-resident stem cells, and fibroblasts) that play key roles in the PAH phenomenon that have yet to be examined.

CONCLUSIONS

Additional studies are needed to better understand the mechanism of cryolipolysis and how it affects the interstitial cells and vasculature. The current study suggests that all cells (interstitial cells and adipose cells) can be affected by the treatment. As reported previously by Stefani, when PAH is suspected after multiple cryolipolysis treatments, cryolipolysis therapy should be discontinued and surgical treatment (liposuction) of the hyperplastic deposits is recommended. Further study is needed to characterize patients who are more at risk of developing an adverse event, and how they can be identified pretreatment.

Acknowledgements

Mr Seaman and Dr Tannan equally contributed to this work. The authors thank Dr Bonnie Straka for her photographic and clinical assistance on this article.

Disclosures

The authors declare no potential conflicts of interest with respect to the research, authorship, and publication of this article.

Funding

This study was partially funded by NIH grant EY022063 to Dr Peirce for support with lab costs and supplies (histology costs, antibodies, and reagents used) and NIH grant T32GM0087 to Mr Seaman as a stipend for tuition.

REFERENCES


